Photoenzymatic Repair of Ultraviolet Damage in DNA

II. *Formation of an enzyme-substrate complex*

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ABSTRACT The photoenzyme from bakers' yeast which repairs ultraviolet-inactivated transforming DNA is mechanically bound to ultraviolet-irradiated DNA in the dark, but not to unirradiated DNA. In the bound condition it is stabilized against inactivation by heat and heavy metals. Both the mechanical binding and stabilization are eliminated by illumination. These observations are consistent with the reaction scheme suggested by kinetic studies, in which the enzyme combines with the ultraviolet lesions in DNA and the complex absorbs light, producing repair and subsequent liberation of the enzyme. The approximately exponential decrease of heat stabilization during illumination gives the first order rate constant for the light-dependent step at the corresponding light intensity. This quantity in turn sets limits on the possible magnitude of the molar absorption coefficient of the enzyme-substrate complex and on the quantum yield of the process.

INTRODUCTION

The preceding paper (1) presented evidence that repair of ultraviolet damage to DNA by the yeast photoreactivating enzyme (YPRE) follows Michaelis-Menten kinetics. In the reaction scheme conventionally associated with these kinetics, the enzyme first combines with its substrate (here, the ultraviolet-induced lesions in DNA) and the resulting complex undergoes a first order reaction which yields the product (repaired DNA) and free enzyme (2):

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow[k_3]{} E + P$$

Kinetic evidence points to the second reaction as the light-requiring step.

If this picture is correct, the enzyme-substrate complex is a stable compound in the dark. It should therefore be possible to demonstrate its existence by gentle fractionation procedures capable of separating unbound enzyme from

DNA, as well as by the altered properties of the enzyme when combined with its substrate. This has been done, as briefly indicated elsewhere (3, 4). The present paper describes the work in detail.

MATERIALS AND METHODS

The procedures and preparations employed for assaying transforming DNA and yeast photoreactivating enzyme are identical with those outlined for the preceding paper (1), with the following exceptions and additions.

The genetic marker C2.5, of *Hemophilus influenzae*, conferring resistance to 2.5 γ/ml of cathomycin (novobiocin), was employed in place of the C25 marker. Exposure of the rather insensitive C2.5 marker to 3500 ergs/mm^2 of 254 mμ ultraviolet reduces its transforming activity to about 20 per cent of the unirradiated value, instead of the 0.3 per cent characteristic of C25.

The calf thymus DNA was prepared by Dr. Roger M. Herriott in this laboratory by a procedure of his own design. Nucleohistone, extracted from finely minced thymus tissue in cold 1 M NaCl, 0.05 M citrate, was precipitated by diluting to 0.15 M NaCl with 0.05 M citrate, and the material was purified by cyclically redissolving in 1 M and precipitating in 0.15 M salt. The purified nucleohistone was dissociated in saturated NaCl and the histone removed by filtration. DNA was precipitated from the filtrate at a 30 per cent final concentration of ethyl alcohol, and redissolved in 0.15 M NaCl, 0.01 M citrate. The final product had an optical extinction for a 1 cm path of $E(260\ m\mu) = 125 \times$ mg N/ml $= 186 \times$ mg P/ml, and $E(260\ m\mu)/E(230\ m\mu) = 2.4$.

Ultracentrifugations were carried out at approximately 5°C in a Spinco model L preparative centrifuge using either the No. 40 angle rotor at 40,000 RPM or the SW-39 swinging bucket rotor at 37,000 RPM as indicated. Sucrose gradients were created in the SW-39 centrifuge tubes by adding successive 1 ml layers of 8 per cent, 6 per cent, 4 per cent, and 2 per cent sucrose in 0.15 M NaCl with a J-tipped pipette, and allowing the tube to stand undisturbed until the obvious layer boundaries blurred out. The sucrose-free sample to be centrifuged was layered over this gradient with the same J-tipped pipette.

Unless otherwise stated, illumination was carried out with a bank of three 20 watt cool white fluorescent lamps in the apparatus previously described (5). "Black-light" fluorescent lamps, used when specified, were the 20 watt General Electric type BLB.

EXPERIMENTAL RESULTS

Detection of the Complex by Ultracentrifugation

The photoreactivating enzyme (PRE), like most proteins, sediments more slowly in an ultracentrifugal field than highly polymerized DNA, and after centrifugation at $\sim$100,000 $\times$ g for a suitable time is found in upper layers of a centrifuge tube from which DNA (in a parallel experiment) is entirely absent. Consequently, upon centrifuging a mixture of the two, the DNA is

expected to sediment away from the PRE, providing they are not bound together. If, however, a stable complex is formed, the PRE should follow the DNA down the tube, and the upper layers should be left free of enzyme.

The result of such an experiment is shown in Fig. 1, where PRE, alone or

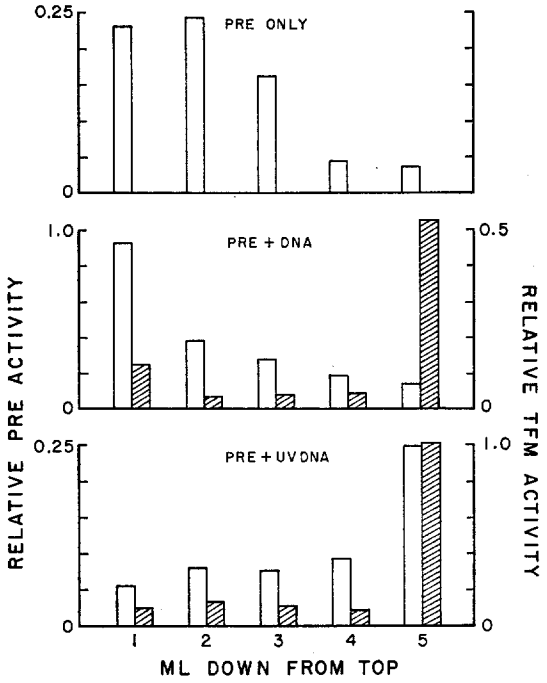

FIGURE 1. Binding of YPRE to ultraviolet-irradiated DNA during sedimentation in the dark. Upper panel, A. S. YPRE (1500 γ/ml in 0.015 M phosphate pH 6.8, 0.15 M NaCl) layered over 4 ml of sucrose gradient and centrifuged 2 hours at 37,000 RPM (84,000 to 149,000 $\times$ *g*) in the Spinco SW-39 swinging bucket rotor. Successive layers tested for enzyme activity (expressed as a fraction of the activity of the uncentrifuged mixture) on UV Sr DNA. Result shows small sedimentation of the enzyme (with possibly slight stirring of the upper layers during extraction of the samples). Middle panel, mixture of 1500 γ/ml YPRE and 2 γ/ml *H. influenzae* C2.5 DNA treated as above. Samples tested for both enzyme and C2.5 transforming activity relative to uncentrifuged control. Result shows independent sedimentation of enzyme (white bars) and DNA (shaded bars). Bottom panel: Same as in middle panel, but using UV C2.5 DNA (3500 ergs/mm^2, 254 mμ). Result shows enzyme sedimenting with the DNA.

mixed with irradiated or unirradiated *H. influenzae* DNA, was layered over a sucrose gradient and centrifuged in a swinging bucket rotor. Successive layers carefully aspirated from the top of each tube after centrifugation were tested for the presence of the DNA by bacterial transformation (using the C2.5 cathomycin resistance marker present in the DNA) and for PRE activity by observing recovery of UV Sr DNA. In the presence of unirradiated C2.5

DNA, PRE sedimented about the same distance as when alone, remaining largely in the upper layers of the tube, but it accompanied irradiated DNA down the tube.

Repetitions of the experiment gave the same distribution pattern shown in Fig. 1, although with variations in the total recovery of both PRE and DNA.

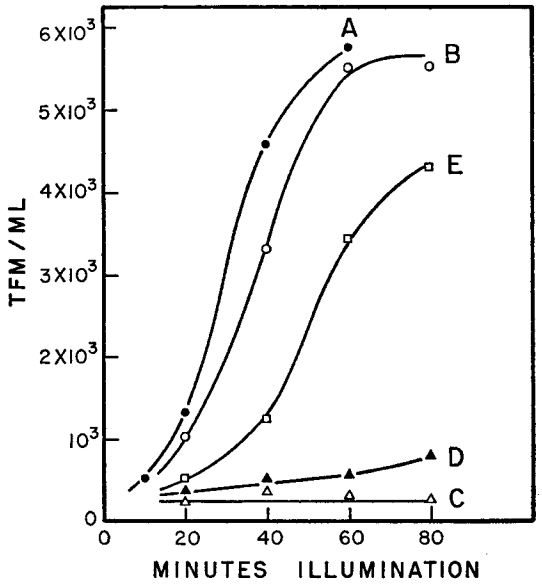

FIGURE 2. Release of YPRE from binding to UV DNA by light. Curve A, 10 ml of five times diluted crude YPRE (giving ~2 mg protein/ml) in 0.12 M NaCl, 0.02 M phosphate pH 6.8, centrifuged 3 hours at 40,000 RPM (100,000 to 145,000 × *g*) in a Spinco No. 40 angle rotor, and the second milliliter from the top of the tube extracted, mixed with UV Sr DNA, and exposed to light. Samples tested for Sr transforming activity at 5×10^{-2} γ/ml UV Sr DNA. Photorecovery shows enzyme activity present. Curve B, same as A, but with 1.8 γ/ml unirradiated calf thymus DNA also added to tube. Shows enzyme activity present. Curve C, same as B, but employing 1.8 γ/ml ultraviolet-irradiated calf thymus DNA (3500 ergs/mm², 254 mμ). Enzyme activity absent. Curve D, same as C, but with 0.18 γ/ml irradiated calf thymus DNA. Enzyme activity largely absent. Curve E, same as C, but mixture illuminated 60 minutes at 37°C before centrifugation. Enzyme activity once more present.

The capacity of UV DNA to bind PRE is eliminated by sufficient exposure of the mixture to light. In the experiment of Fig. 2, mixtures of crude YPRE and calf thymus DNA were centrifuged in an angle head rotor at 110,000 × *g* for 3 hours and samples of the second milliliter down from the top were tested for PR activity on UV Sr DNA. Progressive recovery of Sr transforming activity, showing the presence of active enzyme, occurs with samples from the tube containing PRE only (curve A) or PRE and 1.8 γ/ml unirradiated DNA (curve B) while the corresponding samples from a mixture of PRE and ultra-

violet-irradiated DNA, kept dark, are devoid of enzyme (curve C). This is true even when the UV DNA concentration is reduced to 0.18 γ/ml (curve D). However, with sufficient exposure of the PRE–UV DNA mixture to light before centrifugation, repair of the photoreactivable ultraviolet lesions prevents formation of the complex, and the enzyme is once more left behind upon centrifugation (curve E).

The lost activity in the upper layers of tubes containing UV DNA was not due to competitive inhibition (1), resulting from accidental stirring up of the

TABLE I

INCOMPLETE BINDING OF PHOTOREACTIVATING ENZYME BY LOW CONCENTRATIONS OF IRRADIATED DNA

Mixtures of 265 γ/ml A. S. YPRE and the indicated concentrations of calf thymus DNA and ammonium sulfate (previously titrated to pH 6.8) were made up in 0.09 M NaCl, 0.004 M phosphate pH 6.8 and centrifuged at 5°C in a No. 40 Spinco rotor at 40,000 RPM for 3 hours. The supernatant lying between 1.5 ml and 2.5 ml (measured from the top of each centrifuge tube) was extracted and mixed with one-half volume of 3 γ/ml UV Sr DNA in 0.15 M NaCl and the relative recovery rate under illumination, determined as described in the previous paper (1), used as a measure of PR activity in the supernatant.

Ultraviolet DNA in centrifuged mixture γ/ml.	$(NH_4)_2SO_4$ concentration	PR activity in supernatant
	M	
0	0	1.0*
0.18	0	0.042
0.12	0	0.11
0.09	0	0.30
0.06	0	0.55
0.18	0.06	0.18
0.12	0.06	0.30

*By definition.

sedimented UV DNA during sampling, since it occurred with a DNA concentration too low to produce appreciable inhibition under these assay conditions (*cf.* curve D). Moreover, low activity samples were not enhanced by preillumination before testing on UV Sr DNA, whereas competitive inhibition from irradiated non-transforming DNA can always be reduced or eliminated by allowing the enzyme to act on the competing material first (1).

As would be expected, the amount of PRE left behind increases with decreasing DNA concentration when this concentration is sufficiently low (Table I). About one-twentieth as much PR activity was found in the top of a tube containing 0.18 γ/ml UV DNA as in the corresponding layer from a tube with no DNA, but when the UV DNA concentration was reduced to 0.06 γ/ml, this fraction rose to about one half.

Low concentrations of ammonium sulfate, known to inhibit the photo-

recovery of DNA (1), markedly decrease the capacity of UV DNA to bind PRE.

The above results are all consistent with formation of a stable complex between PRE and UV DNA in the dark, which is decomposed by exposure to light.

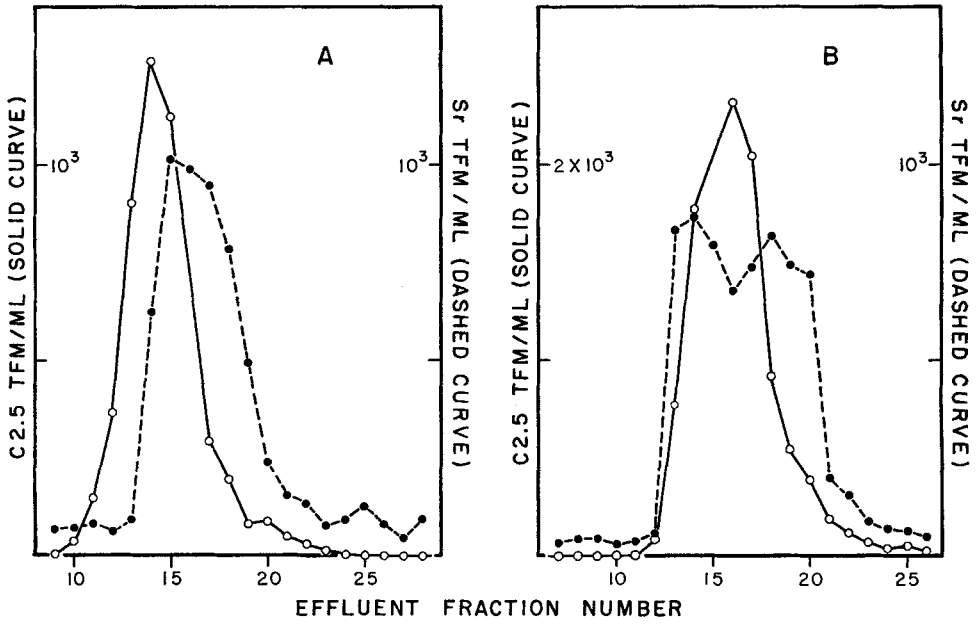

FIGURE 3. Binding of YPRE to UV DNA during passage through sephadex column in the dark. Diagram A, mixture of PRE and unirradiated C2.5 DNA passed through column. DNA, indicated by C2.5 transforming activity (solid curve), appears in effluent before PRE, indicated by rise in Sr transforming activity (dashed curve). Diagram B, same as A, but using ultraviolet-irradiated C2.5 transforming DNA (3500 ergs/mm², 254 mμ). DNA and PRE both appear together in effluent fractions. Irradiated or unirradiated C2.5 DNA (2 γ/ml in 5 per cent glycerol, 0.02 M phosphate pH 6.8, 0.15 M saline plus 2.5×10^{-2} M 2-mercaptoethanol, designated below as "GPS2ME") was mixed with an equal volume of 2650 γ/ml A. S. YPRE in 0.01 M phosphate and 0.5 ml applied at 5°C to a 1 × 15 cm column of sephadex G-75, previously washed with GPS2ME. Material was eluted with GPS2ME and 0.25 ml fractions of the effluent collected. Each fraction was mixed with 0.25 ml UV Sr DNA (at a concentration of 2 γ/ml in diagram A and 1 γ/ml in diagram B), and the mixtures, after illumination at 37°C, were diluted 750-fold and used to transform type Rd *H. influenzae*. The resulting population was assayed for both Sr and C2.5 transformants. The illumination period did not change the transforming activity of unirradiated C2.5 DNA in the fractions of Fig. 3A, but in 3B the ultraviolet-irradiated C2.5 DNA was repaired along with the UV Sr DNA. Because the enzyme and DNA appeared together in B, this repair occurred in all the B fractions. The low ultraviolet sensitivity of the C2.5 marker made the corresponding activity rise only about twofold.

Detection of the Complex by Gel Filtration

Columns of sephadex polysaccharide gel effect fractionation of mixtures on the basis of molecular size (6), sufficiently small molecules diffusing into the gel particles while larger molecules are confined to the interparticle spaces. In passage through the column the larger molecules appear in the effluent before the smaller (with adsorption possibly modifying this behavior in some cases).

When PRE is mixed with unirradiated DNA and passed through a column of sephadex G-75, the first fractions containing DNA always lack PRE, as in Fig. 3A. However, when irradiated DNA is employed, these first fractions also contain PRE as shown in Fig. 3B. This result (obtained in five experiments with unirradiated and four experiments with irradiated DNA) is consistent with a binding of PRE to UV DNA.

Measuring the DNA content of effluent fractions presented no problem, but only very simple measures of enzyme activity could be carried out for all fractions simultaneously. Successive fractions of the column effluent (carrying C2.5 DNA and YPRE) were mixed with UV Sr DNA, exposed to light, and then used to transform competent Rd *H. influenzae* cells, this population being assayed for both the Sr and C2.5 transformants. Under conditions of the assay, C2.5 transforming activity was proportional to the concentration of DNA from the original mixture, permitting direct determination of this quantity. But, although Sr transforming activity was high in the fractions containing active enzyme (which repaired the damaged Sr marker during the illumination) and low in those lacking it, the relation of this transforming activity to enzyme concentration could be determined only by a calibration curve, which varied from experiment to experiment. This in turn changed the over-all appearance of the curves in successive experiments. It had little effect, however, on the tube number at which a perceptible amount of enzyme first appeared because of the steep rise of concentration from tube to tube in this region. Hence, the "leading edge" of each curve is the significant point of comparison.

Stabilization of PRE against Heavy Metals by UV DNA

Many enzymes are more stable in the presence of their substrates than alone (7), presumably because the active site is shielded from chemical attack in the enzyme-substrate complex and because attachment of the substrate helps to maintain the native configuration of the protein. When such stabilization is observed it may indicate formation of a complex.

Yeast PRE is inactivated progressively by parahydroxymercuribenzoate (PHMB) at 37°C, the reaction being stopped but not reversed by an excess

of 2-mercaptoethanol (1). As shown in Fig. 4, such an inactivation by 2×10^{-5} M PHMB is markedly decreased in the presence of ultraviolet-irradiated DNA. The same is true in analogous experiments for inactivation by 5×10^{-6} M Ag^+ (applied for 3 minutes to 46 γ/ml CaP YPRE). Since the concentration

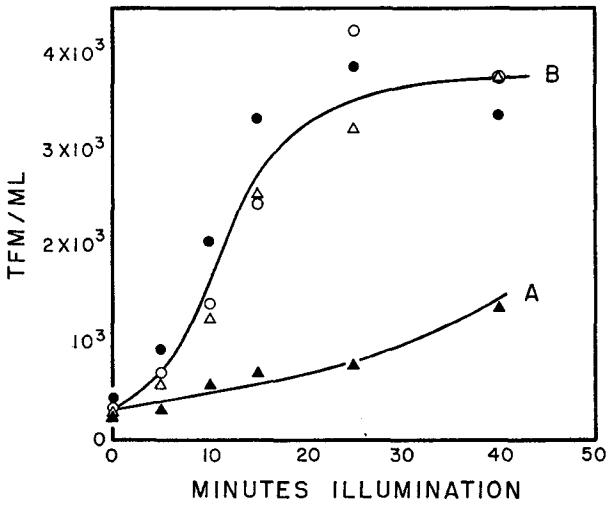

FIGURE 4. Protection of YPRE from parahydroxymercuribenzoate (PHMB) by UV DNA in the dark. Curve A, photoreactivation of UV Sr DNA by enzyme partially inactivated with 2×10^{-5} M PHMB in the presence of unirradiated *H. influenzae* DNA. Curve B, (open triangles) same as A, but with enzyme protected by the presence of UV Sr DNA during PHMB treatment; (open circles) control, enzyme incubated without PHMB in the presence of unirradiated *H. influenzae* DNA; (solid circles) control, enzyme incubated without PHMB in the presence of UV Sr DNA. Duplicate mixtures containing 132 γ/ml A. S. YPRE plus 1.5 γ/ml UV Sr DNA and duplicate mixtures containing the same concentration of A. S. YPRE plus 1.5 γ/ml unirradiated unmarked *H. influenzae* DNA were prewarmed to 36°C. Crystalline sodium PHMB (Sigma Chemical Co.), dissolved in 0.1 M glycylglycine at pH 7.7 and diluted to 2×10^{-4} M in 0.01 M phosphate pH 6.8, was added, 0.11 volume going to one member of each duplicate pair while the other member received the same volume of phosphate. After 15 minutes, all mixtures were made 6×10^{-2} M in 2-mercaptoethanol (Eastman Organic Chemicals) and unirradiated unmarked *H. influenzae* DNA or UV Sr DNA added as required to give an identical final DNA composition for all. Mixtures were then tested for photoreactivation of the UV Sr DNA (1).

of ultraviolet lesions has been estimated as less than 10^{-2} times the concentration of DNA nucleotides (1) (which are present at only 5×10^{-6} M in these mixtures), it is unlikely that any "tying up" of the heavy metals by combination with ultraviolet lesions could afford the observed protection. The latter must be due to a direct interaction of irradiated DNA and enzyme.

Stabilization of PRE against Heat by UV DNA

The activity of A.S. YPRE decreases exponentially with time of heating at

65°C. This inactivation is less extensive in the presence of 0.05 M 2-mercaptoethanol, suggesting that at least part of it is due to accelerated chemical attack on the active site. The rate of inactivation is not changed by adding 0.25 γ/ml of unirradiated DNA, as shown in Fig. 5, but the same concentration of irradiated DNA (3500 ergs/mm^2, 254 mμ) increases the half-life by about an order of magnitude.

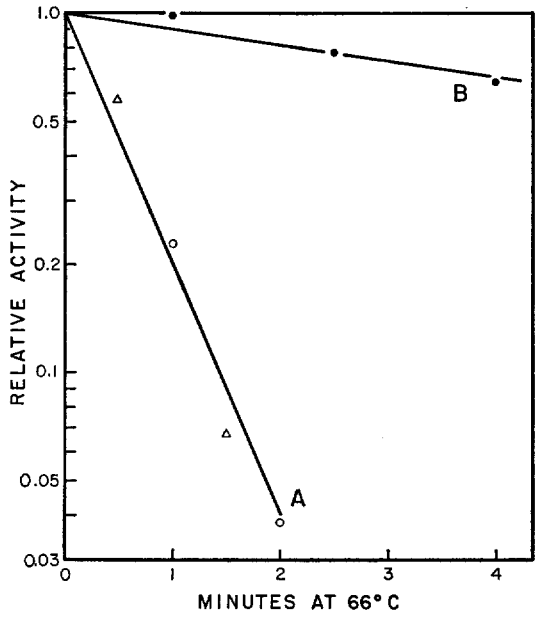

FIGURE 5. Heat inactivation of A. S. YPRE. Curve A, open triangles, A. S .YPRE (265 γ/ml in 0.01 M phosphate pH 6.8, 0.075 M NaCl) heated for the indicated times, cooled, and assayed on UV Sr DNA. Open circles, same as above, but with 0.25 γ/ml unirradiated *H. influenzae* DNA present in addition. Curve B, same as A, but with 0.25 γ/ml ultraviolet-irradiated *H. influenzae* DNA (3500 ergs/mm^2, 254 mμ) present.

The protection from heat is independent of UV DNA concentration when this is sufficiently high, presumably because all the enzyme is complexed with ultraviolet lesions of the DNA. However, at lower concentrations, the protection diminishes with decreasing amounts of UV DNA, as would be expected if some of the enzyme were being "left over" uncombined. This is shown in Fig. 6 where the PR activity in heated PRE–UV DNA mixtures (expressed as a fraction of the activity in identical unheated controls) is plotted *vs.* UV DNA concentration during heating.

Photolysis of the Enzyme-Substrate Complex

The protection from heat afforded by UV DNA is progressively reduced by exposing PRE–UV DNA mixtures to light before heating. This reduction

does not occur if enzyme and DNA are separately illuminated before mixing. At low UV DNA concentrations giving somewhat less than maximum protection, the PR activity initially decreases as an exponential function of preillumination time (Fig. 7).

The mean lifetime τ for this exponential decay (the illumination time required to reduce residual activity to 37 per cent of its value with no illumination) is approximately 1 minute at 37°C when employing the cool white fluorescent tubes used in most of the PR experiments reported to date (5).

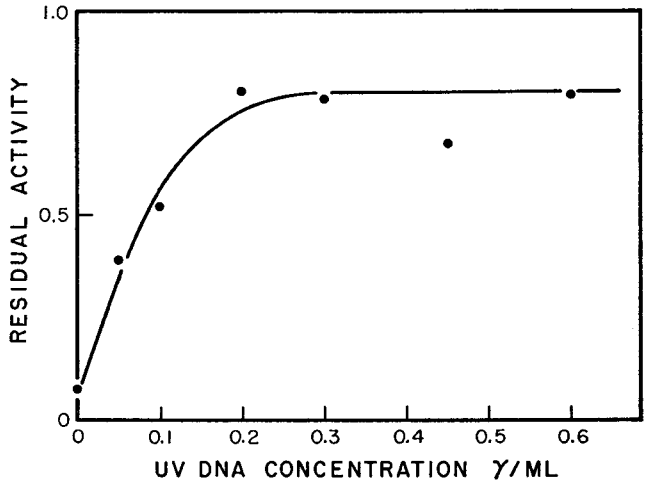

FIGURE 6. Dependence of heat protection of YPRE on concentration of UV DNA. Mixtures of 265 γ/ml A.S.YPRE (in 0.01 M phosphate pH 6.8, 0.075 M NaCl) with varying concentrations of *H. influenzae* DNA were made up in duplicate pairs and one member of each pair heated 2.5 minutes at 65°C. Enzyme activity of each mixture was assayed on UV Sr DNA and residual activity of the heated mixtures expressed as a fraction of that in the unheated controls. Activity in all unheated controls was essentially the same, except at the highest concentrations where perceptible competitive inhibition was produced by the protecting DNA.

With blacklight fluorescent tubes (General Electric Co. type BLB), emitting nearly all their radiation at wavelengths effective for photoreactivation, τ is approximately 10 seconds. These lamps provide approximately 2000 $\mu w/cm^2$ to reaction mixtures in our apparatus, as determined by the ferrioxalate method of Hatchard and Parker (8).

It is readily shown that whenever an enzyme concentration E exceeds its substrate concentration S, and $(E + S) \gg K_m$ (the Michaelis constant) most of the substrate present will be combined with the enzyme as enzyme-substrate complex. Comparison of the A. S. YPRE concentration used in these experiments (440 γ/ml) with those used in Fig. 11 of the preceding paper (1) suggests that the present value of E may approximate 10 K_m, with the sub-

strate in excess. As seen toward the left-hand side of Fig. 6, the activity remaining in heated PRE–UV DNA mixtures, over and above the activity of similarly treated "unprotected" enzyme, is roughly proportional to the concentration of ultraviolet lesions added, in the concentration range applying here. Therefore, this additional "protected" activity should also be proportional to the concentration of enzyme-substrate complex. The observed exponential decay of this quantity upon illumination evidently indicates an approximately first order photolysis of the complex.

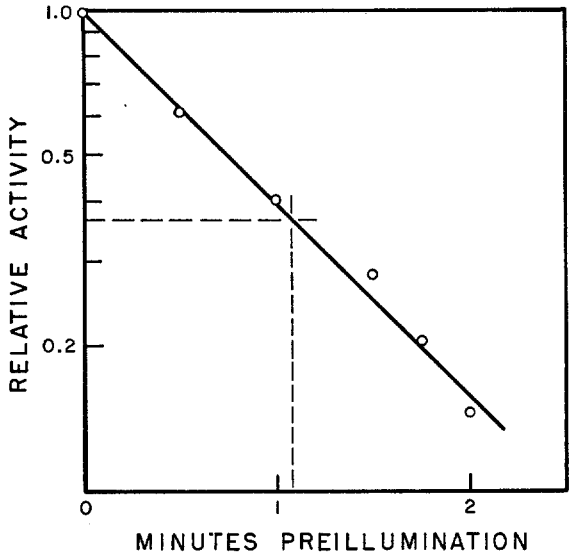

FIGURE 7. Decay of heat protection from UV DNA during illumination of mixtures before heating. Identical mixtures of 440 γ/ml A. S. YPRE and 0.05 γ/ml UV *H. influenzae* DNA (3500 ergs/mm², 254 mμ, in 0.01 M phosphate pH 6.8, 0.075 M NaCl) were illuminated for the indicated times and heated at 65°C for 2.5 minutes. Enzyme activity was assayed on UV Sr DNA and expressed as a fraction of the activity remaining in an unilluminated control. Similar mixtures containing unirradiated *H. influenzae* DNA had negligible activity after heating.

Several details of the phenomenon remain to be understood. In experiments in which appreciable activity remained in controls heated with unirradiated DNA, subtracting this unprotected activity before making the semilogarithmic plot of Fig. 7 straightened out a tendency of the line to curve toward the horizontal. However, even when this correction was applied, a sufficiently prolonged illumination ultimately produced such a bend toward lower slope. This is unexpected from the simple considerations related above. Since the relative magnitudes of E and K_m are only roughly known it may be that the simplifying condition $E + S \gg K_m$ is not well met, even though $E > K_m$. The situation may also be complicated by shifts of equilibrium as the temperature

changes and by changes in K_m as the light is applied and withdrawn, since we are not sure $k_2 \gg k_3$.

PRE has a slightly greater resistance to heat following its illumination alone or in the presence of unirradiated DNA, the half-life at 65°C increasing 20 to 30 per cent after 2 to 3 minutes' cool white fluorescent illumination. The process soon reaches saturation when no further change with light is seen, so that a state of maximum heat resistance can be induced by preliminary illumination of the enzyme preparation. It is not clear whether this represents some intrinsic change in the enzyme, produced by light, or simply the destruction of a photolabile substance in the preparation which is harmful at high temperatures.

Both these phenomena might be clarified by studying the effect of illumination on the protection of PRE from heavy metals. Neither of them affects the magnitude of the initial slope in plots like Fig. 7. Consequently, it is probably safe to consider that the decay of heat protection gives the right order of magnitude for the photolysis rate of the enzyme-substrate complex.

Separate tests show that neither the transforming activity nor the photoreactivability of irradiated DNA is appreciably affected by heating to 65°C for 25 minutes, and that the competitive inhibitory power of irradiated unmarked DNA is unaffected by 40 minutes' exposure to 100°C (12). Hence, the brief heating used here should have no net effect on the ultraviolet lesions or the DNA structure.

DISCUSSION

The foregoing evidence indicates that the photoreactivating agent from bakers' yeast is bound to ultraviolet-irradiated DNA in the dark (but not to unirradiated DNA), and that in this form it is partly protected from inactivation by heat and heavy metals. It is released from this complex by a period of illumination so that the DNA no longer exerts a specific attraction for it. These findings support the indications from kinetic evidence (1) that photoreactivation proceeds by the Michaelis-Menten reaction scheme, with the enzyme first attaching to the photochemical lesion of DNA in the dark, and the repair occurring during subsequent first order photolysis of this enzyme-substrate complex.

Observation of the complex provides an experimentally independent means of detecting photoreactivable lesions in biologically inactive DNA which supplements the competitive inhibition method described in the previous paper. Both methods agree that DNA lacking recognized biological activity can sustain the same type of ultraviolet damage that inactivates transforming DNA, and that this can be repaired both intracellularly and extracellularly by PRE (4).

Competitive inhibition does not interfere with tests which use the complex formation to detect photoreactivable ultraviolet lesions because these tests can be carried out at such low concentrations that inhibition is negligible. However, stabilization of the enzyme by ultraviolet lesions can affect the competitive inhibition test if this is carried out under conditions which partially inactivate the enzyme (7). When sufficiently dilute and pure preparations of YPRE are preincubated or preilluminated with the material under test before adding the irradiated transforming DNA, mixtures in which competing ultraviolet lesions are present sometimes maintain their enzyme activity while mixtures lacking them do not. This differential inactivation tends to cancel the differences in recovery rates caused by competitive inhibition, thereby reducing its apparent magnitude. Such an effect can usually be avoided by properly designed experiments, and can be recognized when it occurs by arranging suitable controls.

As pointed out by Jagger (9), existence of the enzyme-substrate complex offers an explanation of Bowen's findings on the photoreactivation of T2 bacteriophage (10, 11). Bowen concluded that something from the irradiated phage can exist in two forms inside the host cell, passing reversibly from one to the other. In only one of these forms is it susceptible to the reactivating light, undergoing the change which leads to phage recovery. The present work suggests that it is the ultraviolet lesions in phage DNA which may or may not be combined with the proper enzyme inside the host cell. Only in the first case are they subject to photorepair.

The kinetic studies in the previous paper (1) suggested that low concentrations of ammonium sulfate inhibited photoreactivation by interfering with formation of the enzyme-substrate complex. The centrifugation experiments confirm this suggestion directly by showing a smaller binding of enzyme to irradiated DNA in the presence of 0.06 M $(NH_4)_2SO_4$ (Table I).

In order to explain kinetic results with reaction mixtures containing relatively high enzyme/substrate ratios, we assumed that the molar concentration of enzyme in ~50 γ/ml A. S. YPRE exceeded the concentration of lesions in 0.017 γ/ml DNA exposed to 3500 ergs/mm^2 254 mμ radiation (1). This assumption agrees approximately with the results of centrifugation and heating experiments, although the latter indicate only a small margin of excess. If the equilibrium at 5°C favors formation of the enzyme-substrate complex, the enzyme left behind after centrifugation with low concentrations of UV DNA represents approximately the stoichiometric excess over the equivalent quantity of ultraviolet lesions in the mixture. According to Table I, the photoreactivable lesions in 0.06 γ/ml calf thymus DNA exposed to 3500 ergs/mm^2 of 254 mμ radiation will bind about half the enzyme in 265 γ/ml A.S.YPRE; *i.e.*, 2160 γ A.S.YPRE = 1 γ UV DNA (3500 ergs/mm^2). As judged by competitive inhibitory power, calf thymus DNA develops about the same

number of PR lesions as does *H. influenzae* DNA for the same ultraviolet dose (12), so that this estimate for the stoichiometric ratio is applicable to our reaction mixtures.

An independent measure of the same quantity is obtained from Fig. 6, on the assumption that the heat protection begins to decrease with decreasing UV DNA concentration at the point where the enzyme and lesions are approximately equivalent. This assumption gives 1320 γ ASYPRE = 1 γ DNA (3500 ergs/mm^2), within a factor of 2 of the value obtained at 5°C by the centrifugation experiment.

These estimates of the stoichiometric equivalence mean that the turnover number of the enzyme is very low in the experiments carried out to date. The fairly typical reaction mixture whose recovery is shown in Fig. 3A of the preceding paper (1) would contain six to ten PR lesions per enzyme molecule. The bulk of these were repaired in 5 minutes (judging by the disappearance of competitive inhibition in the subsequent test shown in Fig. 3B) so that the turnover rate was around 1 to 2 min.$^{-1}$. This figure is consistent with the decay rate of heat protection shown in Fig. 7, since the turnover number cannot be greater than $1/\tau \approx 1$ min.$^{-1}$. Such values are much lower than in most enzyme reactions, but are entirely consistent with the supposed reaction scheme.

The mean lifetime τ of a photosensitive molecule exposed to a monochromatic energy flux of I_0 μw/cm^2 is

$$\tau = \frac{1}{k_3} = \frac{5.2 \times 10^{10}}{\lambda I_0 \epsilon \phi}$$

seconds, where λ is the wavelength in mμ, ϵ is the molar extinction coefficient, and ϕ is the quantum yield for photolysis (the process being considered as a simple first order reaction with a rate constant k_3). The photoreactivating wavelengths, when using blacklight fluorescent tubes in our apparatus (12), lie between a 340 mμ limit of window transmission and about 400 mμ, averaging ~370 mμ. Using the experimental value for the illumination intensity (2000 μw/cm^2) and the observed τ = 10 seconds, we have $\epsilon\phi \approx 7000$. A comparable figure ($\epsilon\phi \approx 11{,}000$) is obtained with the cool white fluorescent tubes (Fig. 7), estimating the active illumination intensity between 340 and 400 mμ as 200 μw/cm^2 from manufacturer's data (13). This requirement can be satisfied by plausible values of ϵ and ϕ, and also sets certain limits on them.

Since, for our reaction scheme, $\phi \leq 1$, $\epsilon \geq 7{,}000$. Thus the absorption coefficient of the enzyme-substrate complex is at least as great as the 260 mμ absorption of DNA nucleotides. At the other extreme, it is very unlikely that ϵ is as great as 7×10^5, giving $\phi > 10^{-2}$. Therefore the quantum yield is

likewise not extremely small. If our estimates of the concentration of ultraviolet lesions in DNA are of the right order of magnitude (1), this yield is at least as large as the yield for formation of the lesions, and is probably much larger.

The latter finding means that the apparent inefficiency of photoreactivation, as compared with ultraviolet inactivation, is not real on the molecular level. The lower incident energy required for inactivation, as compared with reactivation, arises (*a*) because only a very small fraction of the potential sites of damage in DNA need develop lesions in order to inactivate, while an appreciable fraction of the lesions formed must be repaired to obtain recovery. Hence, the inactivating reaction must proceed only a little way toward "completion" to have its effect, while the reactivating one must proceed a large part of the way. In addition, (*b*) the reactivating illumination is ineffective unless enzyme is combined with the lesion, further reducing the efficiency of photorecovery when not all the lesions are so combined.

As pointed out elsewhere (3), the photoreactivating enzyme system bears a striking formal resemblance to the retinene-opsin system of vertebrate rod vision. This latter system can be considered a photoenzymatic reaction, as stated earlier by Hubbard (14) and recently elaborated in some detail by Wald and Hubbard (15).

The all-trans isomer of retinene (vitamin A aldehyde) can be converted to a mixture of isomers by radiation centering around 385 mμ. Certain of these cis-trans isomers (the so-called *iso-retinene a* and *neo-retinene b*) will combine with the protein opsin to form an enzyme-substrate complex (rhodopsin) in which the absorption band is shifted some 100 mμ toward the red. Illumination at this new wavelength efficiently regenerates the starting material (all-trans retinene) and frees the opsin. Except for the wavelengths involved and the obviously different chemical makeup of the system, this could serve as a perfectly satisfactory model for photoreactivation of DNA.

In both cases part of the chemical change resulting from the absorption of radiation (*i.e.*, from elevation to an excited electronic state) can be reversed by longer wavelength radiation (*i.e.*, elevation to a state lying nearer ground level) provided the initial photoproduct combines with the appropriate protein. This combination, in its excited state, represents the "activated complex" of reaction rate theory (2). In both cases the protein is protected from heat and heavy metal inactivation by its substrate. In the visual system, photodissociation of the enzyme-substrate complex (rhodopsin) is a multistep process, involving low energy dark reactions which follow the light-dependent step (15, 16). This may also be true for photolysis of the PRE-DNA complex, as indicated by its temperature coefficient (1).

The existence of two reactions following a common pattern suggests the

possibility of others. These two examples, therefore, argue that the normal photochemistry of living things, like their other biochemistry, is enzyme-mediated (3). As with other enzymatic reactions, it is conceivable that a given photobiological reaction could occur outside the appropriate protein complex. However, the probability of its occurring and its capacity to win out over competing processes would be markedly changed under these circumstances. As a biological mechanism it requires its enzyme. In this respect, the visual system constitutes the prototype for a more general photobiological process.

The evidence of this and the preceding paper (1) can be accommodated by a simple picture involving a single enzyme and single type of ultraviolet damage, but the possibility of greater complexity is not excluded. A single enzyme may attach to and repair several different types of ultraviolet damage (in the way that opsin, for example, combines with two different isomers of retinene). It is also possible that several similar enzymes are involved, each specific for a different type of ultraviolet lesion. The reaction scheme outlined here is skeletal only, with the specific details remaining to be filled in.

We have now to fill in these details. At this point the outstanding problem is the chemical nature of the ultraviolet lesions in DNA.

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